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Responses of wound-inoculated seedlings of *Pinus elliottii* var. *elliottii* and *Pinus taeda* to mycelial cultures derived from multiple and single basidiospores of *Cronartium quercuum* f. sp. *fusiforme*

T. Miller[1], K.P. Gramacho[1], R.A. Schmidt[1], H.V. Amerson[2] and E.G. Kuhlman[3]

[1]School of Forest Resources and Conservation, Institute of Food and Agricultural Sciences, University of Florida, Gainesville, FL 32611, USA
E-mail: tm1266@aol.com
[2]Department of Forestry, North Carolina State University, Raleigh, NC 27695, USA
[3]Forestry Science Laboratory, Athens, GA 30602, USA

Summary

In 1991, a series of experiments was initiated to examine the effectiveness and research value of inoculating 6-week-old seedlings of slash (*Pinus elliottii* var. *elliottii*) and loblolly pine (*P. taeda*) with suspensions of basidiospores of *Cronartium quercuum* f. sp. *fusiforme* onto wounds made by severing the upper approximately 1 cm of the stems. These experiments were to evaluate the wound technique as an alternative to the standard spray inoculations with basidiospore suspensions, particularly as a means of identifying potential mechanisms of resistance and to facilitate single-spore inoculations. The success of the wound inoculation technique led to experiments in which seedlings of slash pine were wound-inoculated with mycelia from cultures derived from multiplespace and single-basidiospore isolates. These experiments produced what is believed to be the first *in vivo* infection of pine seedlings by single-basidiospore isolates of *C. q. fusiforme*. In the most successful inoculation test, galls were produced on 11 of 58 slash pine seedlings inoculated with six different single-spore isolates. Evaluation of single-spore inoculations was continued with mycelium grown from virulent and avirulent cultures of *C. q. fusiforme*. Fifty seedlings of a half-sib family of loblolly pine were inoculated with each of five single-spore isolates. One V single-spore isolate resulted in galls (8/50). Twelve months after inoculation a cube of tissue was removed from the upper portion of each gall, embedded, sectioned, stained and examined microscopically to evaluate the patterns of colonization and host reactions to the one virulent isolate. Overall, the pattern of colonization was similar to seedlings inoculated with the standard spray technique. However, differences were noted in intensity of colonization and characteristics of spermogonial development.

Key words: basidiospores, inoculation techniques, loblolly pine, slash pine

1 Introduction

Over the past 40 yr, the economic losses in the southern United States (US) to the fusiform rust disease, caused by *Cronartium quercuum* Miy. ex Shirai f. sp. *fusiforme* on *Pinus elliottii* (Engelm.) var. *elliottii* and *P. taeda* L. have been reduced significantly (Pye *et al.* 1997). This success has been largely due to the practical application of the research accomplishments of forest pathologists, forest geneticists and tree breeders in finding the means of reducing the incidence of this destructive disease through deployment of pine families and geographic seed sources with genetic resistance to *C. q. fusiforme*.

In spite of the relative success of using genetic resistance, fusiform rust continues to cause unacceptable losses in many parts of the southern US. Much of this continuing problem may be associated with the pathogenic variability of *C. q. fusiforme* (Snow *et al.* 1975, Powers *et al.* 1977, Kuhlman 1992). Since little is known about the genetics of *C. q. fusiforme*, such research is a prerequisite to progress in understanding the host/parasite interaction and in developing additional strategies for management of the disease.

A major requirement for investigating the genetics of *C. q. fusiforme* is to determine if the *Pinus* host can be infected by a single basidiospore, by mycelium produced from a single basidiospore or with masses of basidiospores, and whether these methods will induce symptoms typical of those occurring in nature.

Research that resulted in the routine production of axenic cultures of *C. q. fusiforme* from multiple basidiospores has been underway for nearly 30 yr (Amerson *et al.* 1985). Success at obtaining a culture from a single basidiospore was first reported in 1984 by Frampton. However, techniques for routinely producing cultures from single basidiospores was reported in 1991 by Hu and Amerson. Their techniques involved casting basidiospores onto cellulose nitrate-acetate membranes that were suspended on media in petri dishes. After small colonies of mycelium were formed the membrane was removed and single basidiospores of *C. q. fusiforme* were transferred to this conditioned "nurse" medium. The medium (HG)was an altered version of that developed by Harvey and Grasham (1974). The final medium (HGYP) consisted of HG plus 0.1 g/L of $CaCO_3$ and 1 g/L each of yeast extract (Y) and peptone (P). Bovine serum albumin (10g/L)(BSA) was added to produce a medium identified as HGYP + BSA. Hu and Amerson (1991) were able to obtain cultures from up to 40% of single basidiospores of *C. q. fusiforme*. The conditioned "nurse" medium was essential for growth of single-spore (SS) cultures.

The SS culture technique of Hu and Amerson (1991) was used by Wang (1991) for inoculation experiments using multispore (MS) and SS cultures to inoculate seedlings of *P. taeda*. The pine seedlings were maintained with their radicles inserted into either modified gresshof and doy solution +Y+P (GDYP) or one-half strength GD. Seedlings were inoculated by placing segments of mycelia with media onto the seedling hypocotyls. Infection of hypocotyls was obtained with MS and SS cultures with and without wounding. There are no reported *in vivo* infections of pine seedlings with SS cultures of *C. q. fusiforme* under greenhouse conditions.

Miller and Matthews (1984) reported that potted 1-yr-old *P. taeda* seedlings became infected and produced typical galls when basidiospores were applied to wounds created by severing the upper portion of the stems. Galls also developed following inoculation of wounds created by pruning limbs from 5-yr-old *P. taeda* seedlings in a plantation.

In 1992 studies were initiated to evaluate the feasibility of routinely inoculating 6-week-old seedlings of *P. elliottii* var. *elliottii* and *P. taeda* through wounds created by severing the upper ca 1 cm from the seedling apices (Miller and Schmidt 1994, 1997). The success of the wound inoculations on seedlings of *P. elliottii* var. *elliottii* and *P. taeda* encouraged us to evaluate the technique to infect pine seedlings with mycelial cultures derived from single basidiospores of *C. q. fusiforme*. Results of recent research using genomic mapping procedures (Wilcox *et al.* 1996) and inoculation studies with virulent (V) and avirulent (A) isolates of *C. q. fusiforme* (Kuhlman *et al.* 1997) have demonstrated a resistant locus or gene (Fr1) in progeny of an open-pollinated family (10-5) of *P. taeda*. The Fr1 resistance in family 10-5 is specific (gene-for-gene), such that V isolates will overcome resistance whereas A isolates should not (Wilcox *et al.* 1996). The avoilability of seed of *P. taeda* family 10-5 and the V and A isolates of *C. quercuum fusiforme* provided us the opportunity to investigate the host-parasite interactions in this system using single-basidiospore cultures developed from the A and V isolates.

This paper summarizes the results of our studies on inoculating *P. elliottii* var. *elliottii* and *P. taeda* seedlings with MS and SS cultures through stem wounds.

2 Material and methods

2.1 Selection and culture of *Pinus* host families

The seedlings of *P. elliottii* var. *elliottii* inoculated in these experiments were from two half-sib families: highly susceptible and susceptible. Seedlings of a single half-sib family of *P. taeda* were inoculated. Seeds of *P. elliottii* var. *elliottii* were germinated, and the germlings were transplanted into leach tubes containing Vermiculite:Perlite:peat moss (3:1:1 by volume). Before inoculation, seedlings were kept in a growth chamber at 25–28° C. Light was from cool-white fluorescent bulbs with a photoperiod of 12 h. Seedlings were fertilized once with a liquid fertilizer (N-P-K, 15-30-15) before inoculation at 6 weeks. The culture of the *P. taeda* seedlings was similar except that seedlings were grown in a greenhouse after transplanting.

2.2 Axenic culture procedures

Collection and processing of basidiospores and growth of cultures are described by Amerson *et al.* (1985). Methods to produce SS cultures are described by Hu and Amerson (1991).

Large quantities of both MS and SS cultures were grown following a procedure used at the Institute of Forest Genetics, Placerville, CA, USA (G. Dupper, pers. comm.). This technique involves blending axenic cultures in GDYP medium for 1 min, and pipetting the resulting suspension into liquid GDYP medium in 250-mL flasks. Liquid cultures were maintained at

20° C without shaking and with no specific light requirement. After several weeks of growth, liquid was decanted, a small portion of fresh liquid medium was added, and the mycelium was homogenized. This suspension was pipetted into HGYP + BSA medium (Hu and Amerson 1991) in 60-mm plastic culture dishes and placed in an incubated chamber at 20–24° C. Maximum mycelial growth was obtained after 2 to 4 months, depending on the volume of mycelial suspension added.

2.3 Source of inocula

The basidiospore inoculum used on seedlings of *P. elliottii* var. *elliottii* was derived from aeciospores collected from galls at several locations in the states of Georgia and Florida. The inoculum for *P. taeda* was derived from virulent and avirulent single-urediniospore isolates of the pathogen from North Carolina and South Carolina (Kuhlman *et al.* 1997). The basidiospores were collected from germinating telia on leaves of *Quercus rubra* L. Single spores were isolated and cultured using a modification of the technique of Amerson *et al.* (1985).

2.4 Pine seedling inoculation

The procedures for inoculating both *P. elliottii* var. *elliottii* and *P. taeda* were the same. Seedlings were inoculated at about age 6 weeks. The apex of each seedling was severed a few millimeters below the base of the plumule with surgical scissors. Inoculum was applied quickly to the freshly cut stem surface. Inoculated seedlings were moved quickly into a mist chamber for periods varying from 24 to 72 h. Seedlings were then returned to a growth chamber or headhouse and finally to a greenhouse. Seedlings were typically observed for symptoms at 3, 6 and 9 months after inoculation.

A series of inoculation tests was initiated in 1993 using MS and SS axenic cultures of *C. q. fusiforme* on seedlings of both *P. elliottii* var. *elliottii* and *P. taeda*. Several different inoculation techniques were tried on wounds on *P. elliottii* var. *elliottii* seedlings.

The basic technique used a segment of mycelium with adhering medium (MAS). Small colonies (clumps) of mycelium (< ca 3 mm diameter) were used intact. Larger colonies were cut into ca 3–4 mm^2 segments using a surface-sterilized razor blade. The mycelial side of the segment was placed on the wound.

To determine if the resin exudate produced at the cut stem surface might inhibit infection, we blotted the exudate with a piece of sterile filter paper just before inoculation. To reduce desiccation of the inoculum segment, the water-holding compound Viterra® (Aglukon Agri-Products, Congers, N.Y., USA) was applied to the wound just before the inoculum. In another method, we tried in an effort to reduce desiccation of the inoculum segment on the seedling by inverting a micropipette tip over the mycelium–agar segment and upper part of the stem.

2.5 Inoculation of *P. taeda* seedlings having the Fr1 locus/gene

Axenic cultures, both MS and SS, were established from basidiospores produced on leaves of *Q. rubra* by *C. q. fusiforme* virulent (V) cultures NC2-36 and avirulent (A) cultures 5C35-5 (Kuhlman *et al.* 1997). Culture procedures yielded 7 SS isolates and 3 MS isolates from the V source and 18 SS isolates and 2 MS isolates from the A source.

Seedlings of the *P. taeda* family 10-5 (Kuhlman *et al.* 1997) were grown to age 8 weeks in a greenhouse at Athens, GA, USA. Sufficient quantities of the V and A cultures of *C. q. fusiforme* were produced to inoculate 324 seedlings: 20 seedlings with each of 2 A MS isolates; 44, 60 and 80 seedlings, respectively, with 3 A SS isolates, SCG, SCD and SCH; and 50 seedlings with each of 2 V SS isolates. The MAS inoculation procedure was used for all seedlings.

After inoculation, seedlings were moved into a humidity chamber at 20° C for 48 h and were kept in a headhouse for 24 h, then moved into a greenhouse. Seedlings were examined for symptoms after 3, 6 and 9 months. Seedlings with galls at nine months were taken to Gainesville, FL, USA, repotted, and placed in a greenhouse. Succulent stem tissues and needle samples were collected from the infected seedlings of family 10-5. The samples were subjected to DNA analysis to establish the presence or absence of the RAPD marker band J7-485A, which is tightly linked with resistance locus/gene Fr1 in family 10-5 (Wilcox *et al.* 1996, Kuhlman *et al.* 1997).

A second experiment with *P. taeda* family 10-5 and A and V cultures was established at the Resistance Screening Center (RSC), US Forest Service, Asheville, NC, USA, in September 1997. Seedlings were grown at the RSC and inoculated at age 10 weeks. The inoculum used was three V SS and two MS isolates and five A SS isolates. The total number of seedlings inoculated was 512, with the number per mycelial culture ranging from eight seedlings to 140 seedlings, depending on the quantity of different cultures. Seedlings were examined for symptoms after nine months. Those with galls were taken to Gainesville, repotted, and placed in a greenhouse.

2.6 Histological observations of infected seedlings of *P. taeda* family 10-5

Fourteen months after inoculation, samples were taken from galls on seedlings of *P. taeda* family 10-5 that were inoculated with V culture NCE. Blocks of tissue about 5 x 10 mm were cut from galls on each of eight seedlings. Samples were fixed in formalin - propionic acid - ethyl alcohol, embedded and sectioned by standard procedures, and stained in Pianeze IIIb (Vaughn 1914).

3 Results

3.1 Infection of seedlings of *P. elliottii* var. *elliottii*

The initial test using MS cultures to inoculate a highly susceptible and a susceptible half-sib family of *P. elliottii* var. *elliottii* was encouraging. Using homogenized mycelia in aqueous suspension or mycelial-agar segments (MAS), the infection of the two families was 22% and 50%, respectively.

Over the next 3 years, we initiated inoculation experiments using both SS and MS axenic cultures of *C. q. fusiforme*. The results of these tests were highly variable (Table 1). Relative to the SS cultures, all cultures infected one or more seedlings in tests 2 and 3. In test 4, only one of the four SS cultures resulted in infection.

Table 1. Results of inoculation experiments on seedlings of *P. elliottii* var. *elliottii* using single-basidiospore (SS) and multi-basidiospore (MS) axenic cultures of *C. q. fusiforme*.

			Axenic cultures			SS cultures			MS cultures	
Test	Date	Host families	SS	MS	Inoculation methods[1]	Number infecting	Seedlings inoculated No.	Seedlings infected No.	Seedlings inoculated No.	Seedlings infected No.
1	1/93	A	7	0	MAS	0	82	0	–	–
		B	7	0	MAS	0	21	0	–	–
2	3/93	A	6	1	MAS	6	38	8	10	2
					MAS + (Vt)	2	20	3	10	0
		B	6	1	MAS	1	17	1	20	2
					MAS + (Vt)	0	13	0	20	3
3	7/94	A	3	0	MAS	3	48	7	–	–
					MAS+B	3	24	5	–	–
4	10/94	A	4	0	MAS	0	48	0	–	–
					MAS+B	0	16	0	–	–
					MAS+T	0	16	0	–	–
					MAS+B	1	16	2	–	–
5	2/95	A	3	1	MAS+B	0	96	0	24	1
6	5/95	A	3	1	MAS+B	0	88	0	22	0

[1] MAS = mycelium-agar segment; Vt = Viterra; B = blot; T = micropipette tip (tube)

The most infection by MS cultures occurred in test 2, where the best results also were obtained with the SS cultures (Table 1). None of the variations in inoculation methods used was an improvement over using entire, small colonies or small segments cut from cultures (Table 1) with the mycelial surface placed onto the stem wound.

3.2 Infection of seedlings of *P. taeda* family 10-5 having the Fr1 locus/gene

At 9 months after inoculation of 324 seedlings of half-sib family 10-5, 11 seedlings had typical fusiform rust galls. Eight of the galls were on the 50 seedlings that were inoculated with a SS culture (NC-E) grown from the virulent *C. q. fusiforme* isolate NC2-36 (Kuhlman *et al.* 1997). An SS culture (NC-B) from the NC2-36 isolate produced a gall on a single seedling of 50. An MS culture (SC-MS-B) grown from the avirulent isolate of *C. q. fusiforme* (SC-35-S) infected and produced galls on 2 of 50 inoculated seedlings of family 10-5.

Of the 11 seedlings with galls that were genotyped, six were positive for the Fr1 locus/gene for resistance and five were negative (Table 2). Both of the seedlings with galls resulting from inoculation with the A MS-SC culture were negative. Among the nine seedlings with galls resulting from the two V cultures, six were positive for the Fr1 locus/gene and three were negative. The DNA profiles of the 11 infected 10-5 seedlings were compared with the profiles of the samples of the family collected from field plots in 1994 (H.V. Amerson, pers. comm.). The analysis showed that the 11 samples were definitely from *P. taeda* family 10-5.

The previous experiment was repeated using three SS and one MS culture of the V isolate NC-2-36 and five SS cultures of the A isolate SC 5-35 to inoculate 512 seedlings of *P. taeda* family 10-5. After nine months, only four seedlings had galls: one from A-SC-SSG, two from V-NC-SSD, and one from V-NCMS. These infected seedlings were not genotyped.

Table 2. Presence (+) or absence (–) of the locus/gene (Fr1) in the infected seedlings of *Pinus taeda* half-sib family 10-5.

Seedling	Inoculum	Source and virulence of SS and MS cultures	Fr-1 locus/gene presence
A	SSNC-E	NC2-36 V	+
B	SSNC-E	NC2-36 V	+
C	MS-SC-B	SC35-5 AV	–
D	SSNC-E	NC2-36 V	–
E	SSNC-E	NC2-36 V	+
F	SSNC-E	NC2-36 V	–
G	SSNC-E	NC2-36 V	–
H	SSNC-E	NC2-36 V	+
I	SSNC-E	NC2-36 V	+
J	SSNC-B	NC2-36 V	+
K	MS-SC-B	SC35-5 AV	–

3.3 Histological analysis of gall tissues from seedlings infected by a virulent, single-spore culture

The outward morphology of the galls produced on the seedlings was quite typical in size and shape for the period after inoculation (Fig. 1).

Examination of the stained thin sections from the samples taken from the eight galls induced by V-SS culture NC-E showed both similarities to and differences from typical fusiform rust galls.

The pattern of colonization of the SS isolate in gall tissue was very similar to that previously reported and illustrated by Jewell *et al.* (1962) and Miller *et al.* (1976) for typical fusiform rust galls. A difference was the less intensive development of the fungus in the areas colonized (Fig. 2). Also, most of the pathogen-induced rays were only a single cell wide (Fig. 3), whereas the rays of fusiform rust galls induced by basidiospores were many cellswide.

A major difference in these galls was the unusual development of the spermogonial layers. Most of the samples from the eight galls had layers of what appeared to be nearly mature spermogonia (Fig. 4A). However, none of these spermogonial had ruptured the epidermis. Also, the cortical cells underlying the spermogonia layers were necrotic, based on the staining reaction, and a periderm had formed between the necrotic cortical cells and the living cortical cells (Fig. 2). A similar zone of darkly stained cortical cells and a newly formed periderm were also apparent even in the seedlings that had not developed spermogonia. In typical fusiform rust galls resulting from infection by masses of basidiospores of *C. q. fusiforme*, spermogonia and underlying cortical cells are identifiable (Fig. 4b). The spermogonia are releasing spermatia through the ruptured epidermis, large quantities of apparently vigorous hyphae are present connecting the spermogonia and the cortical tissues, and the cortical tissue contains large numpers of both hyphae and haustoria.

Figure 1. Fusiform rust galls on seedlings of *Pinus taeda* (family 10-5) at 15 months after wound inoculation with an axenic single-spore culture of a virulent isolate of *Cronartium quercuum* f. sp. *fusiforme*.

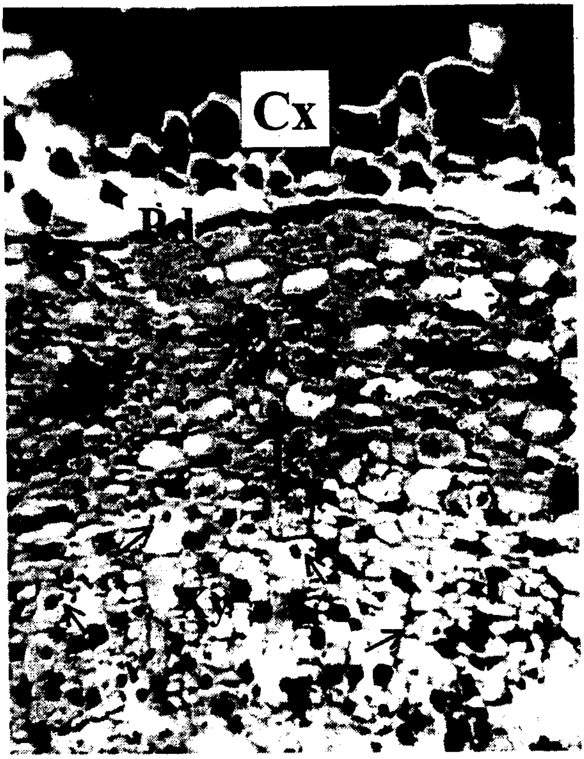

Figure 2. Portion of a fusiform rust gall on seedling of *Pinus taeda* (family 10-5) at 15 months after inoculation showing intensively stained and necrotic cortical cells (Cx), secondary periderm (Pd), and abnormal cells induced by *Cronartium quercuum* f. sp. *fusiforme*. Note the enlarged ray cells (Ry) and abnormal xylem (Xy). Arrows indicate hyphae and haustoria.

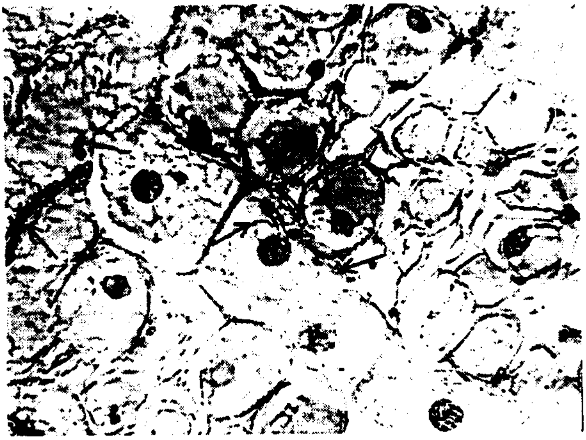

Figure 3. Typical pathogen-induced ray cells in a fusiform rust gall on wound-inoculated seedling of *Pinus taeda* (family 10-5), showing hyphae and haustoria of *Cronartium quercuum* f. sp. *fusiforme*.

Figure 4. Photomicrographs of spermogonia and underlying cortical cells. (A) Galls induced by a single-basidiospore culture of *Cronartium quercuum* f. sp. *fusiforme* showing no release of spermatia and necrosis of cortical cells. (B) Example of a spermogonial layer, release of spermatia, dense areas of hyphae supporting the spermogonia (arrows), and living cortical cells with numerous hyphae and haustoria in a gall produced following inoculation with mass basidiospores.

4 Discussion

We have demonstrated for the first time that it is possible to obtain infection and typical galls on seedlings of *P. elliottii* var. *elliottii* and *P. taeda* with haploid, single genotypes of *C. q. fusiforme*. Fourteen of the cultures grown from single basidiospores of *C. q. fusiforme* induced typical galls on one or more seedlings inoculated through stem wounds. What we can not explain is why such a small percentage of inoculated seedlings was infected and why the results were so variable from test to test.

A completely negative response to the inoculations would clearly indicate either that axenic cultures of *C. q. fusiforme* can not infect wounded tissues of susceptible *Pinus* seedlings or that the inoculation and/or incubation procedures were inappropriate. In fact, few of the inoculation experiments with either *Pinus* species were complete failures. Although pathogen variability among the SS cultures is a possible, a frequent occurrence was infection on 1 or 2 seedlings out of 10–24 inoculated with the same culture. Also, there was a similar inconsistency with the use of MS cultures, a technique that would greatly reduce the probability of the culture being avirulent. Also, the half-sib family of *P. elliottii* var. *elliottii* inoculated most frequently was the most fusiform rust susceptible family we have tested. In fact, this family is consistently infected at over 95%, and frequently has 100% galls with both spray and wound inoculation with mixed basidiospores. These facts would suggest a lack of any genes for resistance.

The results obtained with the inoculation of *P. taeda* half-sib family 10-5 with the SS and MS cultures derived from the V and A isolates of *C. q. fusiforme* were similar to those on *P. elliottii* var. *elliottii*. Only 11 of 324 inoculated seedlings (3%) developed galls after nine months. Eight of those galls resulted from one V SS mycelial culture. However, even in this case the percent gall development was relatively low (8/50 = 16%). Since all 50 seedlings were inoculated at the same time with the same V mycelial culture and incubated under the same conditions, it is difficult to explain the low percentage of gall development.

The second inoculation experiment with V and A SS mycelial cultures on family 10-5 resulted in even less success: only four seedlings out of the 512 inoculated developed galls after nine months. None of the three isolates that infected 10-5 in the first experiment infected any seedlings in this experiment. The 512 seedlings inoculated in the second 10-5 test were far more vigorous and succulent and, based on previous experience, should have been infected.

Inappropriate or ineffective inoculation techniques may account for the lack of consistency in this research. However, as was true with the infectivity of the SS cultures, the same techniques that were successful in one test failed in the next.

The histological examinations of the gall tissue on *P. taeda* family 10-5 are the first of seedlings infected by a single genotype of *C. q. fusiforme* under greenhouse conditions. Although it is inappropriate to make any definitive conclusions from the examination of six galls induced by one single basidiospore isolate on a single half-sib family, the interactions observed on these seedlings warrant further study. One interesting response of the seedlings examined was the formation of the large areas of apparently normal spermogonia that ceased development just before they would normally emerge through the host epidermis. Whether or not this is an artifact of this particular test or an important characteristic of host reactions to a haploid culture of *C. q. fusiforme* should be determined.

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